Table 1
Real-Time PCR Reaction Mixture

Master mix	Volume per reaction	Final concentration
2X QuantiTect SYBR green PCR master mix	25 µL	1X
Forward primer (10 µ*M*)	2.5 µL	0.5 µ*M*
Reverse primer (10 µ*M*)	2.5 µL	0.5 µ*M*
Water	15 µL	NA
Total volume of master mix	45 µL	NA
Template DNA sample (50 µg/mL)	5 µL (250 ng DNA)	5 µg/mL
Total volume of reaction mixture	50 µL	NA

GFP sequence primer set: 5′- CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′- GGT CTT TGC TCA GGG CGG ACT-3′. Internal control cynomolgus β-actin sequence primer set: 5′-CAT TGT CAT GGA CTC TGG CGA CGG-3′ and 5′-CAT CTC CTG CTC GAA GTC TAG GGC-3′. NA, not applicable.

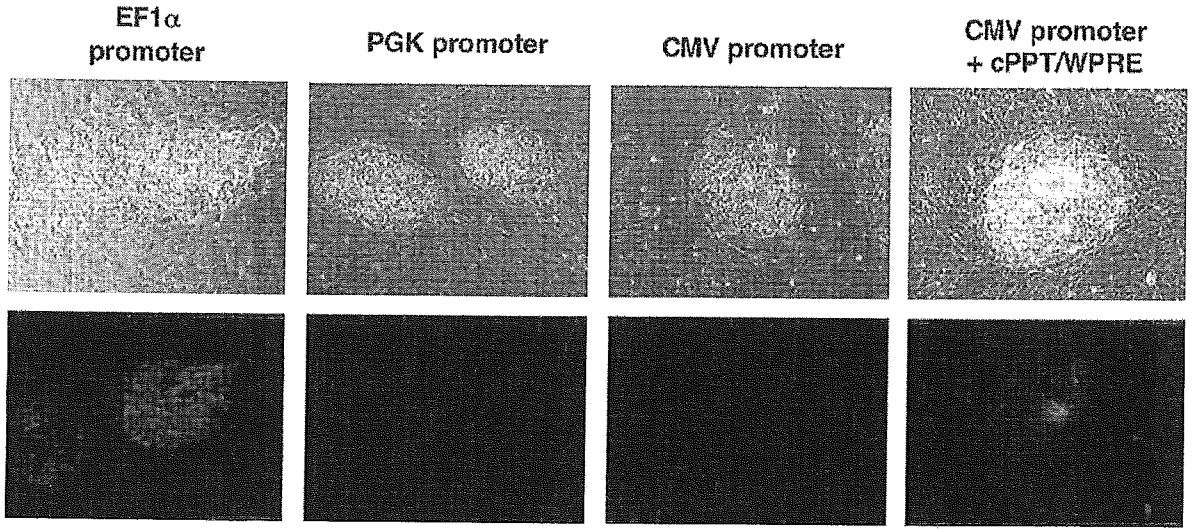

Fig. 2. Promoters and *cis*-acting sequences in simian immunodeficiency virus (SIV) vectors affect transgene expression. Cynomolgus embryonic stem (ES) cells were transduced with green fluorescent protein (GFP)-expressing SIV vectors at 30 TU per target cell. The vectors contain the elongation factor (EF) 1α, phosphoglycerate kinase (PGK), or cytomegalovirus promoter (CMV). The transduced ES cells were observed at d 5 with a fluorescent microscope under a bright field (upper) or dark field (lower). In this cynomolgus ES cell line (CMK6), the usage of the EF1α promoter resulted in the highest GFP expression. In addition, the GFP expression could be enhanced by the inclusion of two *cis*-acting sequences, the central polypurine and termination tract (cPPT) and the woodchuck posttranscriptional regulatory element (WPRE) (rightmost panel).

6. As an ES cell number considerably decreases after passing cells through a strainer before flow cytometry, start experiments with a sufficient number of cells.
7. MEF cells are cotransduced with SIV vector together with ES cells. Therefore, it is suggested to passage transduced ES cells onto untransduced MEF cells several times before DNA

extraction to avoid contamination of transduced MEF cells. In addition, because ES cells are cultured on MEF cells, it is difficult to extract DNA separately from ES or MEF cells. Thus, it is important to know the fraction (percent) of ES cells in total cultured cells (ES plus MEF cells) before DNA extraction in order to calculate the transduction efficiency of ES cells. The fraction (ES vs total cells) can be assessed by flow cytometry (*see* **Subheading 3.3.1.** and **Fig. 1**).

8. We usually use a SYBR green method (Qiagen Quantitect SYBR green PCR kit) rather than a probe method. The former is easier. For the SYBR green method, you do not have to develop specific primers or a probe; rather, regular primer sets are used. It is, however, important to confirm that the PCR does not generate nonspecific bands on an agarose gel because the SYBR green method quantifies all PCR products, including nonspecific ones, if any.
9. The positive control is genomic DNA extracted from cells that contain a known copy number of the target sequence per cell. Dilute the DNA with genomic DNA from naive control monkeys to make a series of diluted positive controls (100, 10, 1, 0.1, 0.01%). The quantitative PCR should be certified each time to yield linear amplifications in the range of the intensity of positive control series (0.01–100%, correlation coefficient >0.98). To certify equal amounts of loaded sample DNA, an internal control sequence (for instance, β-actin) in the same sample should be subjected to real-time PCR. Calculated transduction efficiency (percent) indicates a fraction of cells successfully transduced with SIV vector given that each vector-positive cell contains one copy of the provirus.

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31

In Vivo Tumor Formation From Primate Embryonic Stem Cells

Takayuki Asano, Kyoko Sasaki, Yoshihiro Kitano, Keiji Terao, and Yutaka Hanazono

Summary

To achieve human embryonic stem (ES) cell-based transplantation therapies, allogeneic transplantation models of nonhuman primates would be particularly useful. In this chapter, we describe an example of this model. We prepared cynomolgus ES cells genetically marked with the green fluorescent protein. The cells were transplanted into the allogeneic fetus because the fetus is immunologically premature and does not induce immune responses to transplanted cells. In addition, fetal tissue compartments are rapidly expanding, presumably providing space for engraftment. At 3 mo posttransplantation, a fluorescent teratoma, obviously derived from transplanted ES cells, was found in the fetus. However, transplanted cell progeny were also detected (approx 1%) in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells as assessed by *in situ* polymerase chain reaction. Transplanted cynomolgus ES cells can engraft in allogeneic fetuses. The cells will, however, form a tumor if they "leak" into an improper space, such as the thoracic cavity.

Key Words: Allogeneic transplantation; genetic marking; green fluorescent protein; immunological tolerance; *in situ* PCR; *in utero* transplantation; primate embryonic stem cells; teratoma.

1. Introduction

Because human embryonic stem (ES) cell lines have dual abilities to proliferate indefinitely and differentiate into multiple tissue types *(1,2)*, human ES cell-based transplantation therapies are considered to hold a great potential in the treatment of a variety of diseases and injuries. To address the safety and efficacy of these therapies, allogeneic transplantation models of large animals, especially nonhuman primates, would be useful. However, it has been difficult to transplant primate ES cells or their derivatives into allogeneic hosts. There are two major reasons for this. First, the efficient and stable marking of primate ES cells has been difficult. It is necessary to distinguish transplanted allogeneic ES cell progeny from surrounding host cells. Second, the immune

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rejection of transplanted cells must be circumvented for a sustained engraftment. The cells would otherwise be cleared by immune responses.

We have previously reported highly efficient gene transfer into cynomolgus ES cells using a lentivirus vector derived from the simian immunodeficiency virus *(3)*. Lentiviral transgene expression in ES cells is stable, with minimal levels of transcriptional silencing *(4,5)*. In addition, cynomolgus ES cell sublines stably expressing green fluorescent protein (GFP) were established after electroporation of a GFP-expressing plasmid *(6)*. By using such cynomolgus ES cells genetically modified to express GFP, it is now possible to distinguish transplanted allogeneic ES cell progeny from surrounding host cells as GFP will serve as a good genetic tag.

The early gestational fetus is a good recipient with which to circumvent immune rejection because the immune system is premature *(7,8)*. Furthermore, in the animal fetus, "space" would be relatively available for engraftment as compared to the adult because of the rapid expansion of fetal tissue compartments. Thus, transplanted cells could engraft without conditioning of recipients, such as by irradiation or immunosuppressive treatment.

In this chapter, we show a method to transplant nonhuman primate (cynomolgus macaque) ES cells *(9)* into xenogeneic immunodeficient mice to form teratoma. In addition, we show methods to transplant nonhuman primate (cynomolgus macaque) ES cells stably expressing GFP *(3,6)* into the allogeneic fetus *in utero* and to examine the in vivo fate of transplanted cells using GFP as a genetic tag. At 3 mo after the allogeneic *in utero* transplantation, a fluorescent tumor, obviously derived from transplanted ES cells, was found in the thoracic or abdominal cavity. Notably, transplanted cell progeny were also detected (approx 1%) in multiple fetal tissues. The cells were solitary and indistinguishable from surrounding host cells as assessed by *in situ* polymerase chain reaction (PCR). Thus, transplanted cynomolgus ES cells can engraft in allogeneic fetuses. However, the cells will form a tumor if they "leak" into an improper space, such as the thoracic and abdominal cavities *(10)*.

2. Materials

2.1. Cells

1. Cynomolgus ES cells stably expressing GFP (*see* Chapters 20 and 21, this volume).
2. Mouse embryonic fibroblasts from CD-1 (also referred to as ICR) (Charles River, Wilmington, MA) or BALB/c mice (Charles River).

2.2. Teratoma Formation in Immunodeficient Mice

1. 6- to 8-wk-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratory, Bar Harbor, ME) (*see* **Note 1**).
2. Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA; cat. no.14025-092).
3. Dulbecco's modified Eagle's medium/nutrient mixture F-12 1:1 mixture (DMEM/F12) (Invitrogen, cat. no. 11330-032).
4. ES cell-qualified fetal bovine serum (Invitrogen, cat. no. 10439-024).
5. 10,000 IU/mL penicillin-10,000 µg/mL streptomycin (100X; Invitrogen, cat. no. 15070-063).
6. 200 m*M* L-glutamine (100X; Invitrogen, cat. no. 25030-081).
7. 2-Mercaptoethanol (Sigma, St. Louis, MO; cat. no. M3148).

8. Culture medium for primate ES cells: DMEM/F12 containing 15% ES cell-qualified fetal bovine serum, 2 m*M* L-glutamine, 100 IU/mL penicillin-100 µg/mL streptomycin, and 0.1 m*M* 2-mercaptoethanol.
9. 0.25% trypsin in HBSS (2.5% trypsin 10X liquid; Invitrogen, cat. no. 15090-046).
10. 1% bovine serum albumin (BSA fraction V; Sigma, cat. no. A4503) in HBSS.

2.3. Teratoma Formation in Allogeneic Fetuses

1. Anesthetic and surgical facilities for primates (including ultrasound and inhalation anesthesia equipment) *(11)*.
2. A time-dated pregnant cynomolgus monkey of 50- to 70-d gestation (*see* **Note 2**) *(12)*.
3. Ketamine hydrochloride (Ketalar® 50; Sankyo, Tokyo, Japan).
4. Isoflurane (Forane®; Dainippon Pharmaceutical, Osaka, Japan).
5. A percutaneous transhepatic cholangiography (PTC) needle (22-gage, Sonoguide PTC needle type B; Hakko Medical, Nagano, Japan; cat. no. 22412210).
6. A 1-mL syringe (Terumo, Tokyo, Japan; cat. no. SS-01T) filled with graft cells (10^5–10^7 cells in 200–500 µL).
7. A 1-mL syringe (Terumo, cat. no. SS-01T) filled with normal saline (for flushing).

2.4. Sample Preparation

1. 4% paraformaldehyde (Wako, Osaka, Japan; cat. no. 169-18432) and 8% sucrose (Wako, cat. no. 192-00012) in phosphate-buffered saline (PBS; Invitrogen, cat. no. 10010-023).
2. OCT compound (Tissue Tek series; Sakura, Zoeterwoude, Netherlands; cat. no. 4583) containing 10% sucrose.

2.5. In Situ *PCR*

1. A PTC100 Peltier thermal cycler (MJ Research, Waltham, MA).
2. 20 µg/mL proteinase K (Sigma, cat. no. 39450-01-6) in PBS.
3. 0.1% Triton X-100 (Sigma, cat. no. T8787) in PBS.
4. A slide frame for *in situ* PCR (slide seal; Takara, Shiga, Japan; cat. no. 9066 [25 µL] or cat. no. 9067 [65 µL]).
5. 50 µL Digoxigenin dNTP labeling mix (Roche, Basel, Switzerland; cat. no. 1277065).
6. Rabbit anti-Digoxigenin polyclonal antibody, horseradish peroxidase labeled (Dako, Glostrup, Denmark; cat. no. P5104) diluted (1:100) in 2% BSA and 5% horse serum (Invitrogen, cat. no. 16050-130) in PBS.
7. A Vector SG substrate kit (Vector, Burlingame, CA; cat. no. SK-4700).
8. Kernechtrot solution (0.1% Kernechtrot in aluminum sulfate; Muto, Tokyo, Japan; cat. no. 4087).

3. Methods

3.1. Teratoma Formation in Immunodeficient Mice

1. Wash ES cells with HBSS twice and add 0.25% trypsin to the dish at 37°C for 3 min. Neutralize trypsin with ES culture medium and make a suspension of ES cell clumps.
2. Transfer the cell suspension into a 50-mL conical tube, centrifuge it at 140*g* for 4 min, and resuspend the pellet with 20 mL 1% BSA/HBSS.
3. Centrifuge the cell suspension again at 140*g* for 4 min and resuspend the pellet with an appropriate volume of 1% BSA/HBSS (10^6 cells in 150–200 µL per injection site).
4. Aspirate the ES cell suspension into a 1-mL syringe with a 23-gage needle and inject the suspension into NOD/SCID mice subcutaneously (*see* **Note 3**).

5. Resulting tumors will be palpable at 8–13 wk after the injection. Expose, observe, and excise tumors.
6. Fix tumor samples (5 × 5 × 3 mm) at 4°C for 4 h in 4% paraformaldehyde and 8% sucrose in PBS and embed the samples in paraffin for histological examination. To prepare fresh frozen samples, embed samples (5 × 5 × 3 mm) in OCT compound containing 10% sucrose, freeze them in liquid nitrogen, and store them at −80°C.

3.2. Teratoma Formation in Allogeneic Fetuses

3.2.1. Anesthesia

1. Prepare a pregnant monkey around the end of first trimester (50–70 d; full term 165 d) (*see* **Note 2**).
2. Give the monkey 10 mg/kg ketamine hydrochloride intramuscularly. Secure the monkey on a table and monitor maternal heart rate by electrocardiography (*see* **Note 4**).
3. Induce and maintain anesthesia by inhalation of isoflurane (1.5–2%) mixed with 100% oxygen via a mask.

3.2.2. In Utero *Transplantation*

1. Shave whole abdomen and sterilize the surface with iodine solution (from **Subheading 3.2.1., step 3**).
2. Determine fetal position by transabdominal ultrasound with a 7.5-MHz convex probe (*see* **Note 5**).
3. Let an assistant secure the other side of the uterus while an operator holds the transducer parallel to the intended course of the needle.
4. Select an optimal entry site into the uterine cavity, avoiding the placental tissue.
5. Insert a 23-gage PTC needle through the maternal skin and uterine wall into the amniotic cavity and then into the desired site (e.g., peritoneal cavity, brain, or liver) under continuous ultrasound guidance (*see* **Note 6** and **Fig. 1**). A small push of an injector can visualize a tip of the needle on echocardiography.
6. Let an assistant gently inject the cells (200–500 µL) and flush the needle with 100 µL normal saline. The operator should focus on keeping the tip of the needle in an appropriate position.
7. Confirm adequate heart beats after the procedure (*see* **Note 7**).

3.2.3. Caesarian Section

1. Prepare the pregnant monkey after transplantation as described in **Subheading 3.2.1.** *In utero* transplantation is usually done around the end of the first trimester (50–60 d) (*see* **Subheading 3.2.2.**). The full term is 165 d; therefore, the *in utero* incubation time of transplanted ES cells is about 3 mo.
2. Expose the gravid uterus through a midline incision and deliver the fetus through a low transverse hysterotomy (*see* **Note 8**).
3. Clamp and divide the cord. Remove the placenta and cord. Close the uterus and abdomen with absorbable sutures.
4. Insert a small catheter (24-gage intravenous catheter) into the umbilical vein and irrigate the newborn with normal saline to completely wash out fetal blood for mercy killing. Open the chest and abdomen, observe the whole body, and excise tumors (*see* **Fig. 2A–C**). Collect tissues.
5. Fix tissue samples (5 × 5 × 3 mm) at 4°C for 4 h in 4% paraformaldehyde and 8% sucrose in PBS and embed the samples in paraffin for histological examination. To prepare fresh

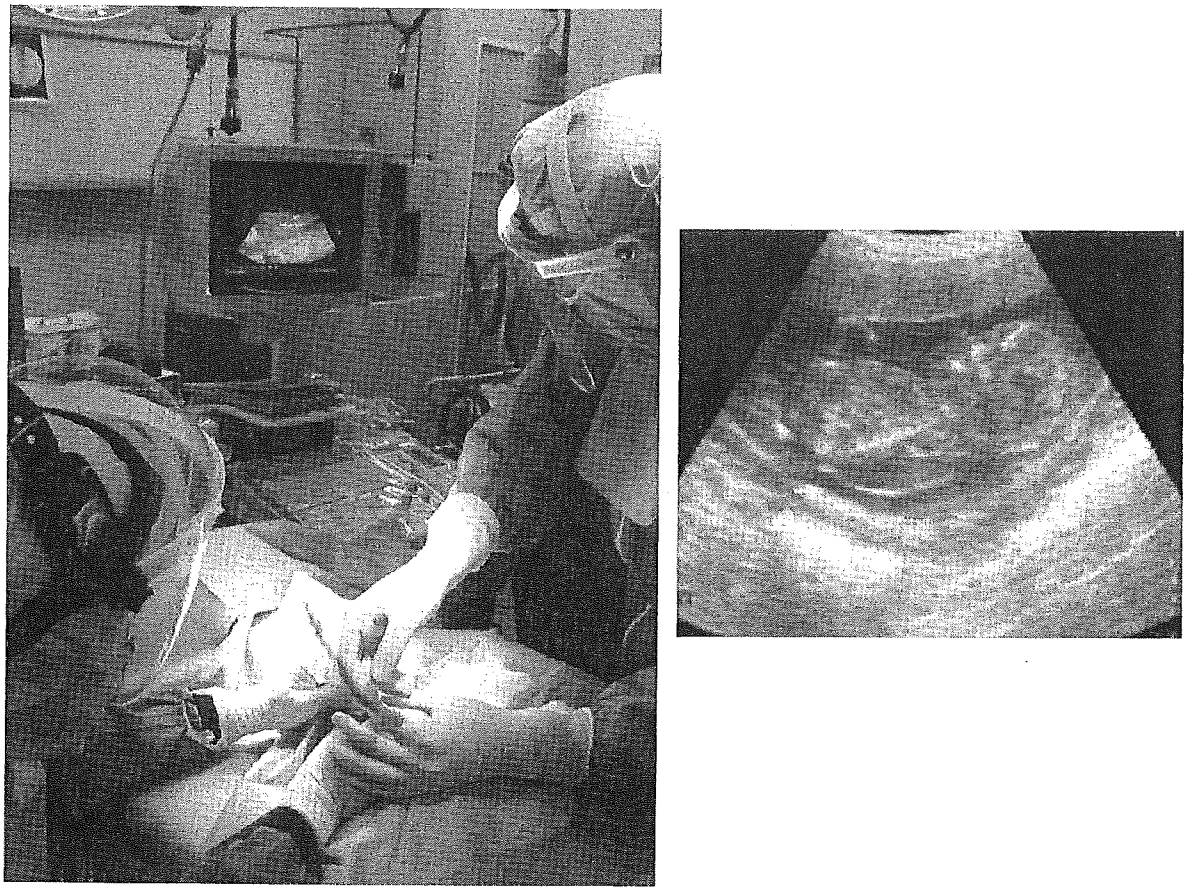

Fig. 1. *In utero* transplantation. Pregnant monkeys were anesthetized by intramuscular administration of ketamine hydrochloride (Ketalar). Cynomolgus ES cells genetically modified to express GFP (10^6 cells/fetus) were injected into the fetal abdominal cavity or liver through a 23-gage needle using an ultrasound-guided technique around the end of the first trimester (**left**). The full term is 165 d. The weight of the fetus at the time of transplantation was estimated at 20 g, which is equivalent to that of an adult mouse (**right**).

frozen samples, embed samples ($5 \times 5 \times 3$ mm) in OCT compound containing 10% sucrose, freeze them in liquid nitrogen, and store them at −80°C.

3.3. In Situ *Detection of Transplanted Cell Progeny*

You may examine tissue sections for in vivo fate of transplanted cell progeny by *in situ* PCR, which amplifies marker (GFP) sequences ***(10,13)***. It is especially useful when it is difficult to identify cells by staining specific surface markers, when GFP fluorescence is hampered by the high autofluorescence of tissue samples, or when the transgene expression is shut down ("silenced") in vivo.

3.3.1. Cell Wall Permeabilization

1. (Optional) If a tissue section is embedded in paraffin, then dewax it by dipping the slide in xylene three times, each for 10 min, and then in 100% ethanol three times, each for 10 min. Air-dry the slide.
2. Soak the slide in 20 µg/mL proteinase K/PBS and incubate it at 37°C for 10 min (*see* **Note 9**).

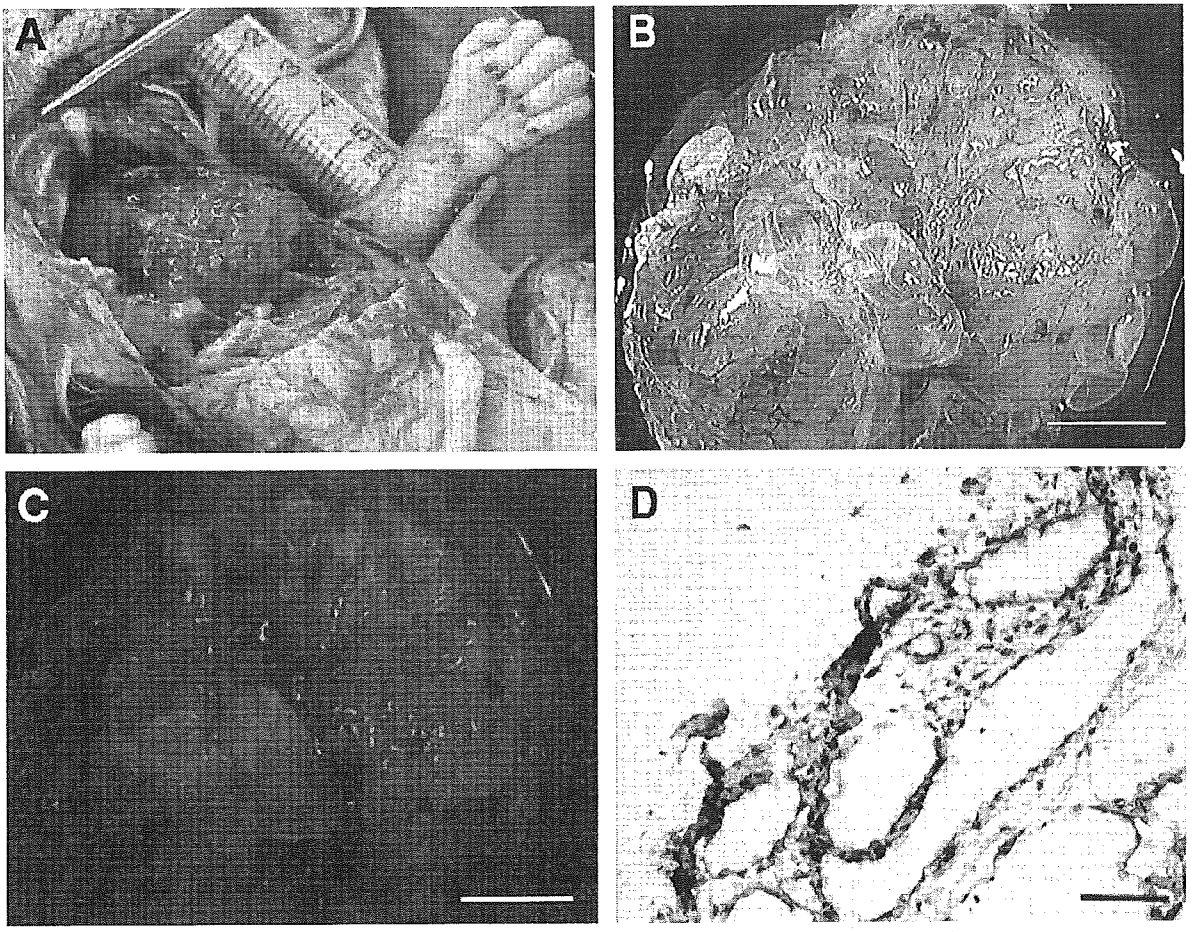

Fig. 2. Teratoma formation in a cynomolgus fetus after transplantation of allogeneic ES cells. (**A**) A tumor (4 × 3 × 2.5 cm) was detected in the thoracic cavity 3 mo after allogeneic transplantation of ES cells expressing GFP. The tumor was observed in (**B**) a bright field and (**C**) a dark field (**C**) under a fluorescence stereomicroscope. GFP was expressed in the tumor, clearly indicating that the tumor was derived from transplanted ES cells. (**D**) The GFP gene was also detected in the tumor cells by *in situ* PCR (stained black). Bar in **B** and **C** = 1 cm. Bar in **D** = 50 μm.

3. Soak the slide in 0.1% Triton X-100/PBS for 5 min and wash it with PBS twice, each for 5 min.
4. Soak the slide in 95% ethanol for 10 min and then in 100% ethanol twice, each for 10 min, to remove proteins and air-dry it.

3.3.2. In Situ *PCR*

1. Attach a slide frame to the slide (from **Subheading 3.3.1., step 4**) and incubate it at 95°C for 5 min.
2. Apply a master mix of *in situ* PCR to the slide at room temperature (*see* **Note 10** and **Table 1**).
3. Cover the slide with a film (*see* **Note 11**).
4. Place the slide upside down in a PTC100 Peltier thermal cycler and start a cycling program (i.e., 94°C for 1 min and 55°C for 2 min with 15 cycles; *see* **Note 12**).

3.3.3. Detection

1. Remove the slide frame gently after PCR (from **Subheading 3.3.2., step 4**) and soak the slide in two changes of PBS for 5 min each.
2 Dropwise add an horseradish peroxidase-labeled anti-Digoxigenin solution (diluted 1:100 with 2% BSA and 5% horse serum in PBS) onto the slide and incubate it at 37°C for 2 h.

Table 1
***In Situ* PCR Reaction Mixture**

Master mix	Volume per reaction	Final concentration
10X PCR buffer (Mg^{2+} free)	2.5 μL	1X
25 m*M* $MgCl_2$	4.5 μL	4.5 m*M*
dNTPs mixture (2.5 m*M* each)	3 μL	420 μ*M* dATP
Digoxigenin (DIG) DNA labeling mix (Roche)	3 μL	420 μ*M* dCTP
		420 μ*M* dGTP
		378 μ*M* dTTP
		42 μ*M* DIG-dUTP
Forward primer (10 μ*M*)	2 μL	0.8 μ*M*
Reverse primer (10 μ*M*)	2 μL	0.8 μ*M*
Takara Taq polymerase (5 U/μL)	0.8 μL	0.16 U/μL
Water	7.2 μL	NA
Total volume of master mix	25 μL	NA

NA, not applicable.

Primer set for the GFP sequence: 5′-CGT CCA GGA GCG CAC CAT CTT C-3′ and 5′-GGT CTT TGC TCA GGG CGG ACT-3′.

3. Soak the slide in two changes of PBS for 5 min each.
4. Dropwise add a Vector SG solution onto the slide, stain it for 3–10 min, and gently wash it with water for 10 min.
5. Dropwise add a Kernechtrot solution and incubate the slide at room temperature for 1–2 min to stain nucleotides and gently wash it with water for 10 min.
6. Mount the slide with glycerol and observe it under a light microscope (*see* **Note 13** and **Fig. 2D**).

4. Notes

1. Although we use NOD/SCID mice (lack of B and T lymphocytes but presence of natural killer cells), SCID mice are usually used in many other laboratories to form teratomas from ES cells. NOD/SCID mice are more highly immunodeficient than SCID mice; thus, NOD/SCID mice may be better in the setting of xenotransplantation.
2. Cynomolgus or rhesus monkeys are the most appropriate to work with because of ES cell availability and their size. In the monkey fetus, "the window of opportunity" for successful tolerance induction may be earlier and narrower than thought *(14)*. To avoid immune responses, transplantation at earlier days (around 40–50 d) may be better.
3. It is not necessary to disperse ES cell clumps to single cells when transplanting ES cells into mice (or other animals). We transplanted about 1×10^6 ES cells (corresponding to two confluent 60-mm dishes) per site in mice. There is, however, considerable variation among reports: from 10–15 clumps (200 cells) per site *(2)* to 5×10^6 cells per site *(15)*. ES cells are usually transplanted subcutaneously into the hind leg muscle, testis capsule, or abdominal cavity. In our experiments, a teratoma was formed in any site. It is recommended to choose injection sites you can observe easily from the outside and from which you can easily excise tumors.
4. For ultrasound-guided transplantation operations, endotracheal intubation is not necessary.
5. We prefer a small convex transducer rather than a big linear transducer because of the small size of the monkey fetus. Although a needle adapter is available, we prefer the freehand technique.

6. You may puncture transplacentally when the placenta is located anteriorly. Bleeding from the placenta usually stops spontaneously. However, we recommend every effort to avoid this approach by manipulation.
7. The survival rate with this *in utero* transplantation technique is currently 100%, excluding those fetuses that died from massive teratoma formation.
8. Uterine atony requiring oxytocin administration is quite rare in primates.
9. The treatment with proteinase K may need longer time depending on samples.
10. The amount of master mix per slide is 25 μL for Takara cat. no. 9066 and 65 μL for cat. no. 9067.
11. Slides are attached to the Takara slide seal kit. Be careful not to trap air under films.
12. The PCR conditions should be optimized for each *in situ* PCR.
13. The results should be observed within the same day. On the following day, the tissue would peel off, making examination difficult.

Acknowledgments

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VII

Animal Models and Therapy

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Safe And Efficient Collection of Cytokine-Mobilized Peripheral Blood Cells From Cynomolgus Monkeys (Macaca fascicularis) with Human Newborn-Equivalent Body Weights

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Abstract: *Hematopoietic stem cells in bone marrow can be mobilized into peripheral blood by cytokine administration. Cytokine-mobilized peripheral blood stem cells are of great use in clinical applications. We previously established a modified procedure for the collection of cytokine-mobilized peripheral blood cells from rhesus monkeys (*Macaca mulata*) using a commercially available apparatus originally developed for human subjects. In this study, we examined the efficacy and safety of this method with even smaller macaques, cynomolgus monkeys (*Macaca fascicularis*), which are equivalent to human newborns in body weight (mean = 3.3 kg). Using the manufacturer's unmodified protocol (n=6), one monkey died of cardiac failure and three developed severe anemia. In contrast, using our modified procedure (n=6), no such complication was observed in any animal. In addition, the harvested nuclear cell, mononuclear cell and CD34$^+$ cell counts were significantly higher with the modified method. The modified method should allow safe and efficient collection of cytokine-mobilized peripheral blood cells from non-human primates as small as human newborns in a non-invasive manner.*

Key words: *cynomolgus monkey, cytokine mobilization, leukapheresis, peripheral blood stem cell*

Introduction

Although hematopoietic stem cells (HSCs) usually reside in the bone marrow, they can be mobilized into the peripheral blood by the administration of cytokines such as granulocyte colony-stimulating factor (G-CSF) [24]. Cytokine-mobilized peripheral blood stem cells are widely used for autologous and allogeneic trans-

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Table 1. Hematological analysis of cynomolgus monkeys after cytokine treatment

Animals	Sex	Age (years)	Body weight (kg)	After cytokine treatment				
				White blood cells (10^2/μl)	Red blood cells (10^4/μl)	Hemoglobin (g/dl)	Hematocrit (%)	Platelets (10^4/μl)
Unmodified procedure								
292049	Male	6	4.6	680	632	12.0	41.4	36.1
293051	Female	6	2.5	1091	535	10.7	39.0	35.7
292079	Female	7	3.2	514	480	11.6	36.5	35.8
292238	Female	7	3.2	548	653	13.4	44.4	30.0
394029	Female	5	3.2	355	590	11.7	42.3	42.4
296116	Male	3	3.1	361	583	12.0	43.8	26.6
Average		5.7	3.3	592	579	11.9	41.2	34.4
Modified procedure								
001046	Female	3	3.5	872	484	12.4	38.9	43.7
001045	Male	3	3.3	519	415	9.7	33.4	44.2
001049	Male	3	3.5	434	501	12.3	38.7	43.8
001053	Male	3	2.6	802	456	10.3	35.1	57.7
001047	Male	4	3.3	805	438	11.1	35.3	40.4
398042	Male	5	3.8	887	521	11.9	36.8	38.4
Average		3.5	3.3	720	469	11.3	36.3	44.7

plantation therapies to treat hematological malignancies such as leukemia and lymphoma [16, 17]. The cells have also been intensively studied as a donor source of stem cells for gene and cell therapies [11, 20, 30]. An efficient method for collecting cytokine-mobilized peripheral blood cells in monkeys would facilitate such studies in a clinically relevant manner.

The procedure for collecting peripheral blood cells from living animals is referred to as leukapheresis: peripheral blood is withdrawn, nuclear cells are removed, and the rest of the blood is returned [7]. Automatic instruments for this procedure are commercially available [23, 27]. The removed cells are then enriched for a stem cell fraction such as $CD34^+$ or $AC133^+$ cells for clinical applications [5, 29]. Although leukapheresis is widely conducted for human adults, it is difficult to apply to regular experimental macaque monkeys because of their small size. Generally speaking, the procedures involved preclude the application of leukapheresis to animals weighing less than 10 kg in body weight [18].

We and others have previously reported leukapheresis procedures for non-human primates with body weights of less than 10 kg using rhesus monkeys (*Macaca mulata*, average 7 kg) [3, 9]. In such small animals, acute cardiac failure due to the relatively large extracorporeal blood flow is a critical adverse event which can occur during leukapheresis. To avoid this, we modified the procedure by reducing the extracorporeal blood volume as much as possible and adjusting the withdrawal speed frequently in response to the results of real-time monitoring of hemoglobin (Hb) and hematocrit (Ht) values [3]. In this study, we examined the efficacy and safety of our procedure using even smaller non-human primates, cynomolgus monkeys (*Macaca fascicularis*, average 3.3 kg), which have body weights equivalent to human newborns.

Materials and Methods

Animals

Twelve cynomolgus monkeys (*Macaca fascicularis*) (3–7 years old, 2.5–4.6 kg) bred at the Tsukuba Primate Research Center (Ibaraki, Japan) were enrolled in this study (Table 1). Animals were free of intestinal parasites, herpes-B, simian type-D retrovirus and simian varicella virus. All monkeys were housed indoors at 23–27°C and 50–70% humidity with 12 air changes per hour and a 12-h/12-h light/dark cycle. Animals were individually housed in stainless steel cages and fed 70 g of commercial monkey chow (Type AS; Oriental Yeast, Chiba, Japan) and 200 g of fruit daily. All monkeys were healthy as assessed by annual examinations. This

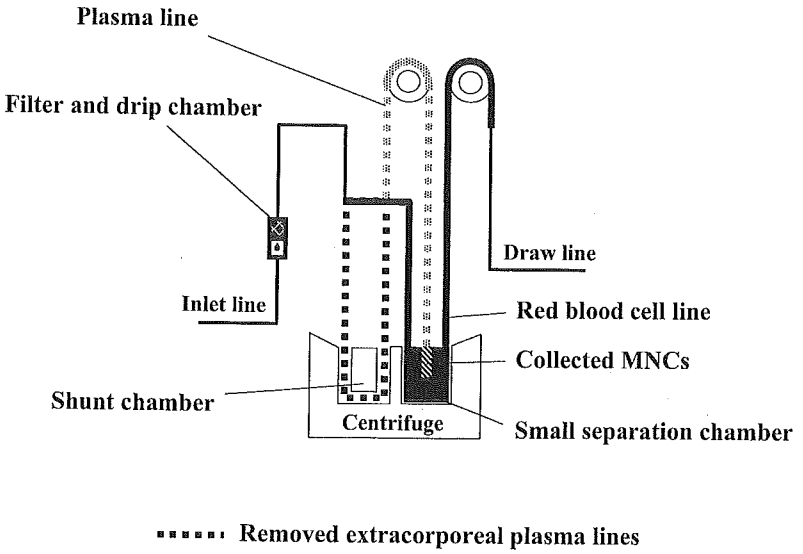

Fig. 1. Diagram of the apheresis kit after the modifications. In the modified system, a small separation chamber (S25A) was installed in the standard apheresis kit and the extracorporeal blood lines were shortened to reduce the extracorporeal blood volume from 130 to 70 ml. MNCs, mononuclear cells.

study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [12] and the Guiding Principles for Animal Experiments Using Non-human Primates formulated by the Primate Society of Japan [21]. This study was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases.

Apparatus

A CS3000 blood separator (Baxter, Deerfield, IL, USA) was used as described previously [3]. Briefly, a standard apheresis kit was installed in the CS3000 blood separator. The smallest separation chamber (S25A) in the kit was used. To reduce the extracorporeal blood volume, the plasma line of the standard apheresis kit was cut away and the red blood cell (RBC) line was directly connected to the inlet line using a polypropylene tube connector (Iuchi, Osaka, Japan) under sterile conditions, bypassing the shunt chamber (Fig. 1). In addition, the regular inlet and draw lines were replaced with lines shorter in length and smaller in diameter (extension tube, 70 cm, 1.4 ml, 2.5 mm diameter; TOP, Tokyo, Japan) to further reduce the extracorporeal blood volume.

Preparative regimen

During the three weeks preceding leukapheresis, a total of 60–90 ml of autologous blood was obtained from each monkey as described previously [6]. The collected autologous blood was supplemented with acid citrate dextrose (ACD) and stored at 4°C prior to use. Recombinant human (rh) stem cell factor (SCF, 50 μg/kg; Amgen, Thousand Oaks, CA, USA) and rhG-CSF (50 μg/kg; Chugai, Tokyo, Japan) were administered to animals subcutaneously daily during the 5 days preceding leukaphersis [9]. On the day of leukapheresis, the right or left femoral artery was cannulated using a 5-Fr polyurethane catheter (Anthron PU; Toray, Tokyo, Japan). The saphenous vein was also catheterized with a 19-gauge intracatheter (Terumo, Tokyo, Japan). This cannulation was performed under general anesthesia with the administration of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan).

Leukapheresis

All leukapheresis procedures were performed under general anesthesia with endotracheal intubation (Fig. 2). Vital signs were monitored with electrocardiography, blood pressure, oxygen saturation and respiration. Animals received a dose of 100 U/kg heparin (Aventis Pharma, Frankfurt, Germany) just before the initiation of leukapheresis. The plasma flowed directly into the inlet line bypassing the shunt chamber (Fig. 1). The inlet line

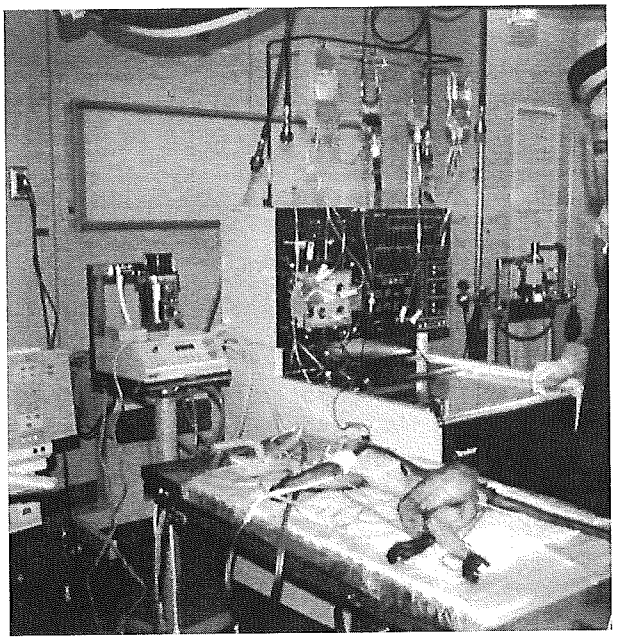

Fig. 2. Leukapheresis procedure. Cynomolgus monkeys were intubated and all procedures were performed under general anesthesia with monitoring of vital signs. The body weights (3.3 kg on average, see Table 1) were similar to those of human newborns.

was connected to an intracatheter placed in the saphenous vein of the animal. The draw line was connected to a catheter in the femoral artery just before starting the procedure. The apheresis kit was filled with the autologous blood collected as described above.

After the leukapheresis was completed, the blood remaining in the apheresis kit was recovered and either used to fill another apheresis kit or returned into the animal. Immediately after the leukapheresis, animals were given an appropriate dose (10 mg per 1,000 U heparin) of protamine sulfate (Aventis Pharma) to neutralize heparin. Animals also received a course of 0.5 mg/kg butorphanol tartrate (Bristol-Myers Squibb, New York, NY, USA) intramuscularly for 3 days to alleviate any post-operative pain.

Analysis of leukapheresis products

The product obtained during leukapheresis was collected in the S25A separation chamber. The product (40–45 ml) was mixed with 7 ml of ACD. The recovered RBCs, white blood cells (WBCs), mononuclear cells and platelets were enumerated with a Sysmex K-4500 instrument (Toa-iyoudenshi, Kobe, Japan). Hb and Ht values were also examined with this instrument. Although the instrument was originally developed for human blood samples, we have confirmed that it works properly for monkey blood samples. Blood cells were collected after centrifugation at 1,200 rpm for 10 min and suspended in the ACK buffer (Biosource, Camarillo, CA, USA) for the lysis of RBCs. $CD34^+$ cells were isolated with immunomagnetic beads conjugated to a monoclonal antibody clone 561 (Dynal, Lake Success, NY, USA) that reacts to both human and cynomolgus CD34 [26, 33]. The harvested $CD34^+$ cells were counted. CD34 is a clinically-relevant cell-surface marker of HSCs, and $CD34^+$ cell transplantation is widely performed as HSC transplantation in patients with cancer or other disorders [5, 19].

Results

We examined the safety and efficacy of leukapheresis using the manufacturer's protocol (n=6) and our modified version (n=6) in cynomolgus monkeys (Fig. 2). In both groups, we administered G-CSF and SCF to monkeys for 5 days to mobilize HSCs into the peripheral blood. The administration resulted in an increase in peripheral WBC counts to 66,000 cells/μl on average (Table 1), and was not associated with any adverse effect such as fever or anorexia. There was no significant difference in the increase in the peripheral WBC counts after cytokine treatment between the unmodified and modified procedure groups. In the modified protocol, a small separation chamber (S25A) was installed instead of the regular one in the blood separator, and the extracorporeal blood lines in the standard apheresis kit were shortened [3, 9, 22]. As a result, the extracorporeal blood volume was reduced from 130 to 70 ml (Fig. 1). In both groups, blood was processed at a rate of 10–12 ml/min and the total processed volume was two to three times the estimated total blood volume [4] of each animal (Table 2). In the modified protocol, every time the processed blood volume increased by 50 ml/kg, a 1-ml blood sample was collected *via* the draw line, and Hb and Ht values were examined throughout the procedure to adjust the plasma pump speed [14]. The plasma pump speed was increased when Hb and Ht values decreased. Conversely, it was decreased when Hb and Ht values increased. In addition, when Hb and Ht values increased, normal saline

Table 2. Leukapheresis procedures

Animals	Estimated total blood volume (ml)*	Processed blood		Complications
		Total volume (ml)	ml/kg	
Unmodified procedure				
292049	293	600	130	None
293051	217	600	240	Severe anemia
292079	231	400	125	Severe anemia Died of cardiac failure
292238	231	600	188	None
394029	231	700	219	Severe anemia
296116	227	600	194	None
Average	238	583	183	
Modified procedure				
001046	237	750	214	None
001045	236	600	182	None
001049	244	800	229	None
001053	205	500	192	None
001047	236	600	182	None
398042	258	700	183	None
Average	236	658	197	

*The total blood volume was estimated with the following formula [4]. For males, (Total blood volume, ml) = 44.07 × (Body weight, kg) + 90.25. For females, (Total blood volume, ml) = 19.95 × (Body weight, kg) + 167.24.

was infused *via* the inlet line for volume replacement.

After the completion of the unmodified procedure (n=6), one animal died of acute cardiac failure and three animals developed severe anemia (Hb<8.0 g/dl, Fig. 3). In contrast, none of the animals that underwent the modified procedure (n=6) developed cardiac failure or severe anemia (Table 2). The Hb and Ht values were significantly better preserved during the modified procedure (Fig. 3). In addition, the numbers of harvested nuclear cells, mononuclear cells, and $CD34^+$ cells were significantly increased with the modified procedure compared to the unmodified one (Fig. 4).

The leukapheresis products were contaminated with considerable amounts of RBCs and platelets, when the apparatus was operated in automatic mode under the unmodified protocol. In the modified version, we performed manual adjustment of the plasma pump speed in response to the results of the real-time monitoring of Hb and Ht values during the leukapheresis as described above, and successfully reduced the contamination (data not shown). The reduction in contaminated RBCs also contributed to the amelioration of anemia after leukapheresis in the modified procedure group. No microbial contamination was detected in cultures of the leukapheresis products from the unmodified or modified procedures.

Discussion

In this paper, we reported leukapheresis in cynomolgus monkeys. Our modified protocol significantly improved mononuclear and $CD34^+$ cell harvest compared to the manufacturer's protocol. Under our modified procedure, we routinely collected 5×10^6 $CD34^+$ cells per kg, which is equivalent to numbers in human trials published in the literature [13, 25, 28, 31]. Thus, with our modified protocol, it is possible to collect sufficient numbers of $CD34^+$ stem cells for various applications including transplantation experiments in monkeys. In fact, we achieved successful hematopoietic reconstitution in myeloablated cynomolgus monkeys after the autologous transplantation of $CD34^+$ cells obtained with this procedure [2]. Of note, this procedure can be safely and effectively applied to monkeys with small body weights (2.6–3.8 kg), equivalent to those of human newborns. Although numerous clinical trials have demonstrated the safety and effectiveness

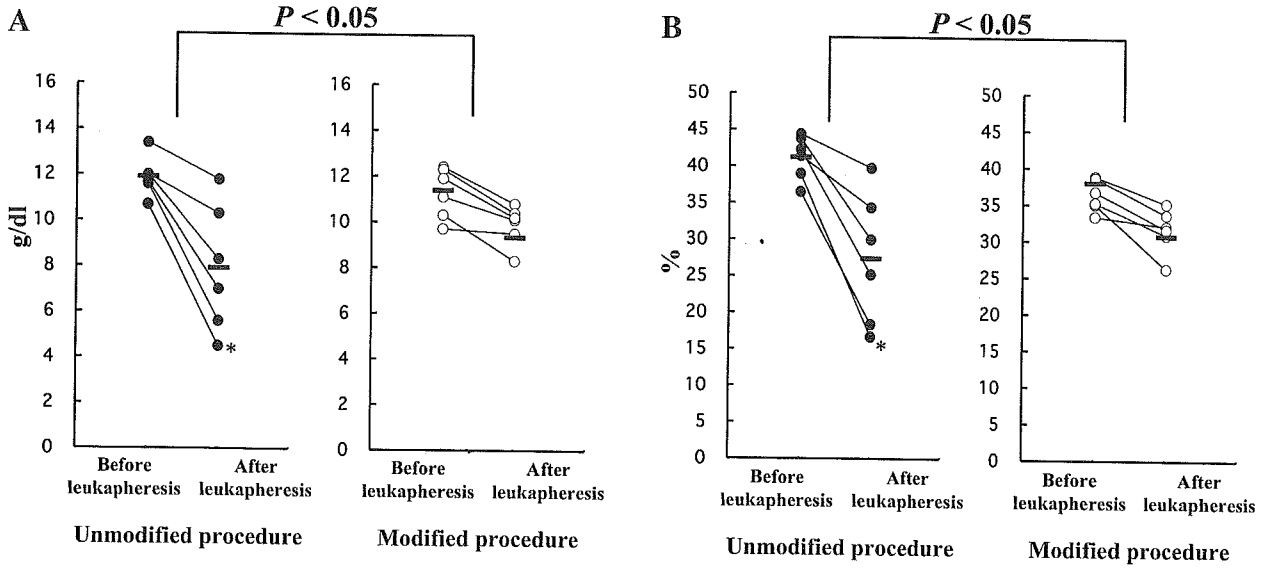

Fig. 3. Avoidance of severe anemia with the modified procedure. The degree of anemia was significantly ameliorated with the modified procedure as compared to the unmodified one as assessed by the ratios of hemoglobin (A) and hematocrit levels (B) after versus before the leukapheresis. One monkey died of cardiac failure (*, 292079) after the unmodified procedure. A: Hemoglobin levels. B: Hematocrit levels.

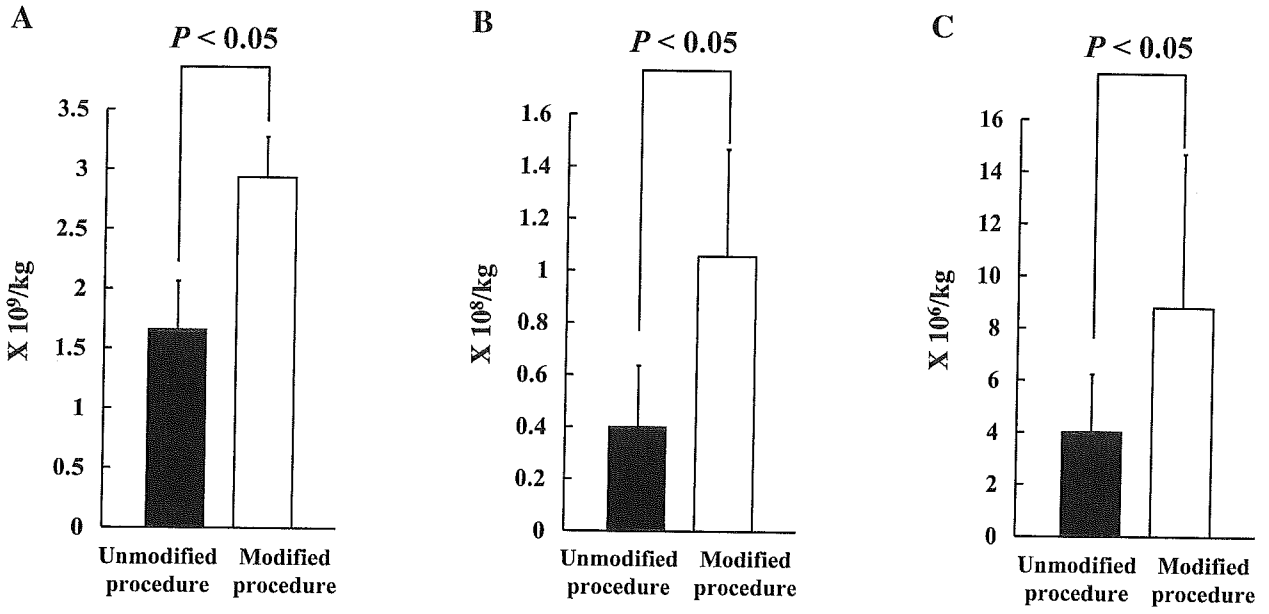

Fig. 4. Larger harvest of cells with the modified procedure. The numbers of harvested nuclear cells (A), mononuclear cells (B), and $CD34^+$ cells (C) were significantly increased with the modified procedure. A: Total nuclear cells. B: Mononuclear cells. C: $CD34^+$ cells.

of leukapheresis for adults [10, 13, 25, 28] and children [1, 8, 15, 31], only one very limited trial has been conducted on a human newborn baby [32]. To our knowledge, this paper is the first systematic documentation of leukapheresis for small primates.

One of the most serious complications in leukapheresis with small animal subjects is cardiac failure due to the relatively large amount of extracorporeal blood volume [10, 32]. The main symptoms include hypotension and dyspnea, which sometimes result in

death, *e.g.* animal 292079 (Table 2). To avoid this complication, the extracorporeal blood volume should be reduced as much as possible. No monkeys underwent cardiac failure after our modified procedure. There was, however, an age variation in the unmodified and modified procedure groups. The unmodified procedure group included higher age animals (6 and 7 years). Cynomolgus monkeys of these ages are young adults, and presumably they are more resistant to stress or invasion than monkeys of a juvenile age (3 years). Clearly, the age distribution of the groups, the higher age monkeys belonging to the modified procedure group, was not better than the present way.

Non-human primate models would be useful for preclinical studies of cell and gene therapies. We have previously reported the transplant of $CD34^+$ stem cells into the ischemic myocardium in cynomolgus monkeys and found that the cardiac function was improved, indicating that further investigation is warranted for clinical application of $CD34^+$ stem cell transplant to such disorder [34]. In other studies, we successfully transplanted gene-modified $CD34^+$ stem cells into cynomolgus monkeys as a preclinical gene therapy [11, 30]. In this way, our safe and efficient method for collecting peripheral blood stem cells should allow investigators to develop and test new therapies using stem cells in small non-human primates.

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